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(54) Title: NUCLEIC ACID ANALOGS WITH A CHELATING FUNCTIONALITY			
(57) Abstract			
<p>A nucleic acid analog comprising a polymeric strand which includes a sequence of ligands bound to a backbone made up of linked backbone moieties, which analog is capable of hybridization to a nucleic acid of complementary sequence, further comprising a chelating moiety capable of binding at least one metal ion by chelation.</p> <div style="text-align: right; font-size: 1.5em; margin-top: 100px;">Folio → Dr. Ørum</div>			

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Folio → Dr. Brown

NUCLEIC ACID ANALOGS WITH A CHELATING FUNCTIONALITY

The present invention relates to nucleic acid analogs having a chelation functionality, to their uses in assay procedures, to methods of capturing them to solid supports and to methods of concentrating solutions of them.

Nucleic acid analogs having important new utilities in assay procedures and in the field of diagnostics have been described in WO 92/20703. These nucleic acid analogs had a number of new properties making them of special importance in the field of diagnostics as well as in the field of antisense therapeutics.

They typically feature a polyamide backbone bearing a sequence of ligands which are nucleic acid bases. The analogs described there have the property of hybridizing with great specificity and stability to natural nucleic acids of complementary sequence.

In order to aid the detection and the manipulation of such nucleic acid analogs in diagnostics or other assay procedures and the like operations, it is desirable to provide the nucleic acid analogs with detectable labels. It is also desirable to find ways of capturing said nucleic acid analogs to solid supports. Various labels are described in WO 92/20703. Also, the capture of the nucleic acid analogs to solid supports via bound nucleic acid or nucleic acid analog sequences acting as capture probes is described.

However, it is desirable to find alternative capture methods and in particular methods which do not require a tailored capture probe which is sequence specific but rather are generally applicable to such nucleic acid analogs.

In EP-A-0 097 373 the synthesis of nucleic acids labeled with a complexing agent is described. However, the synthesis of these compounds appears to be complicated.

Furthermore, whilst natural nucleic acids are readily and routinely concentrated by precipitation from solution by ethanol, centrifugation and resuspension, no such convenient method presently exists to aid those working with these nucleic acid analogs.

The present invention now provides according to a first aspect thereof a nucleic acid analog comprising a polymeric strand which includes a sequence of ligands bound to a backbone made up of linked backbone moieties, which analog is capable of hybridization to a nucleic acid of complementary sequence, further comprising,

nucleobases, aromatic moieties, DNA intercalators, nucleobase-binding groups, heterocyclic moieties, reporter ligands and chelating moieties;

each of C^1-C^n is $(CR^6R^7)_y$ (preferably CR^6R^7 , CHR^6CHR^7 or $CR^6R^7CH_2$) where R^6 is hydrogen and R^7 is selected from the group consisting of the side chains of naturally occurring alpha amino acids, or R^6 and R^7 are independently selected from the group consisting of hydrogen, (C_2-C_6) alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C_1-C_6) alkoxy, (C_1-C_6) alkylthio, NR^3R^4 and SR^5 , where R^3 and R^4 are as defined below, and R^5 is hydrogen, (C_1-C_6) alkyl, hydroxy, alkoxy, or alkylthio-substituted $(C_1$ to $C_6)$ alkyl or R^6 and R^7 taken together complete an alicyclic or heterocyclic system;

each of D^1-D^n is $(CR^6R^7)_z$ (preferably CR^6R^7 , CHR^6CHR^7 or $CH_2CR^6R^7$) where R^6 and R^7 are as defined above;

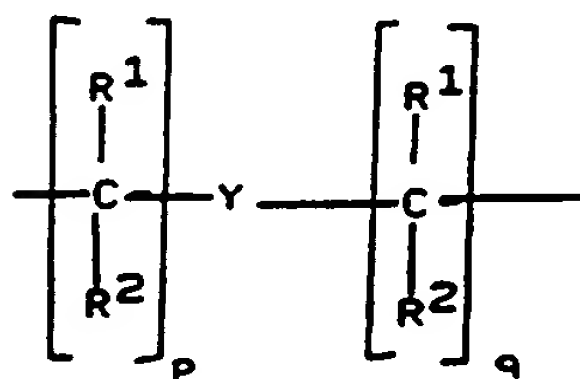
each of y and z is zero or an integer from 1 to 10, the sum $y + z$ being at least 2, preferably greater than 2, but not more than 10;

each of G^1-G^{n-1} is $-NR^3CO-$, $-NR^3C^5-$, $-NR^3SO-$ or $-NR^3SO_2-$, in other orientation, where R^3 is as defined below;

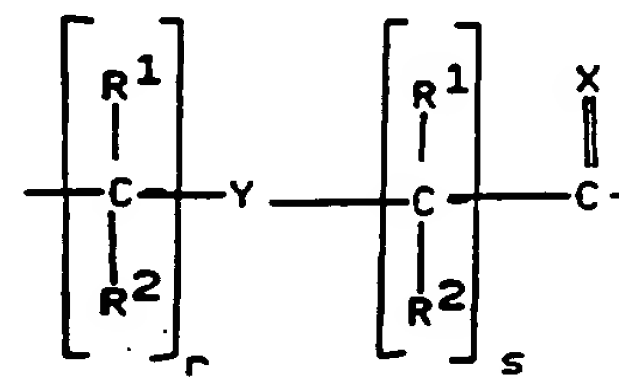
each of A^1-A^n and B^1-B^n are selected such that:

(a) A is a group of formula (IIa), (IIb), (IIc) or (IId), and B is N or R^3N^+ ; or

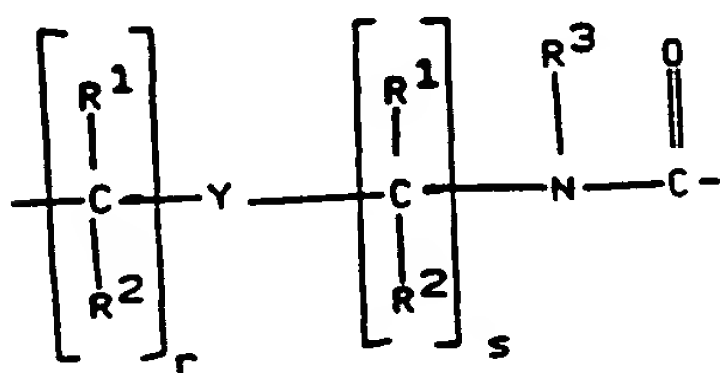
(b) A is a group of formula (IId) and B is CH ;



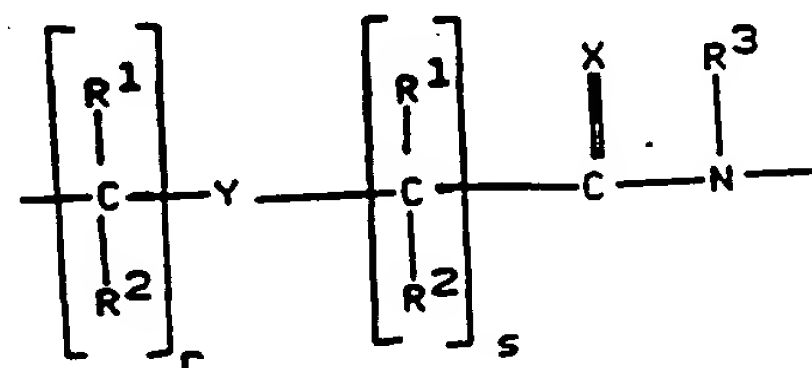
Formula 2



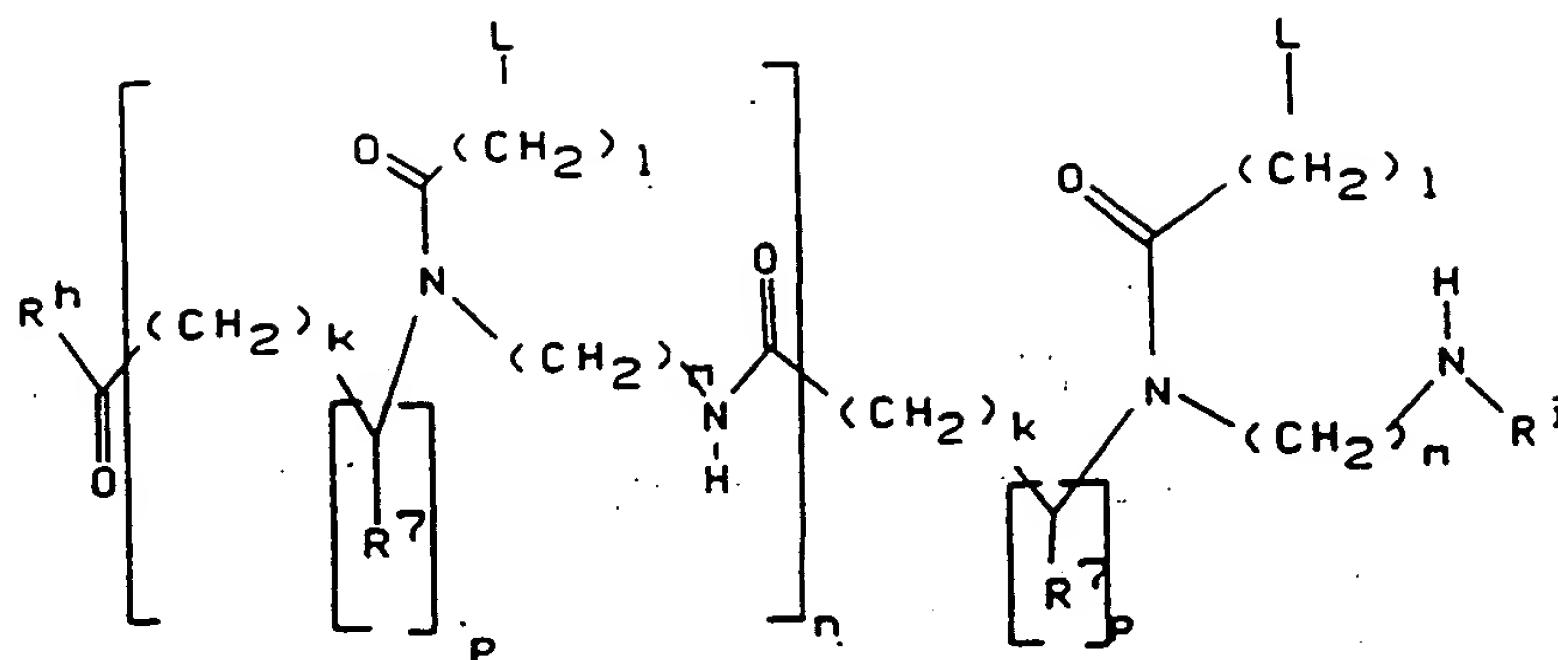
Formula IIb



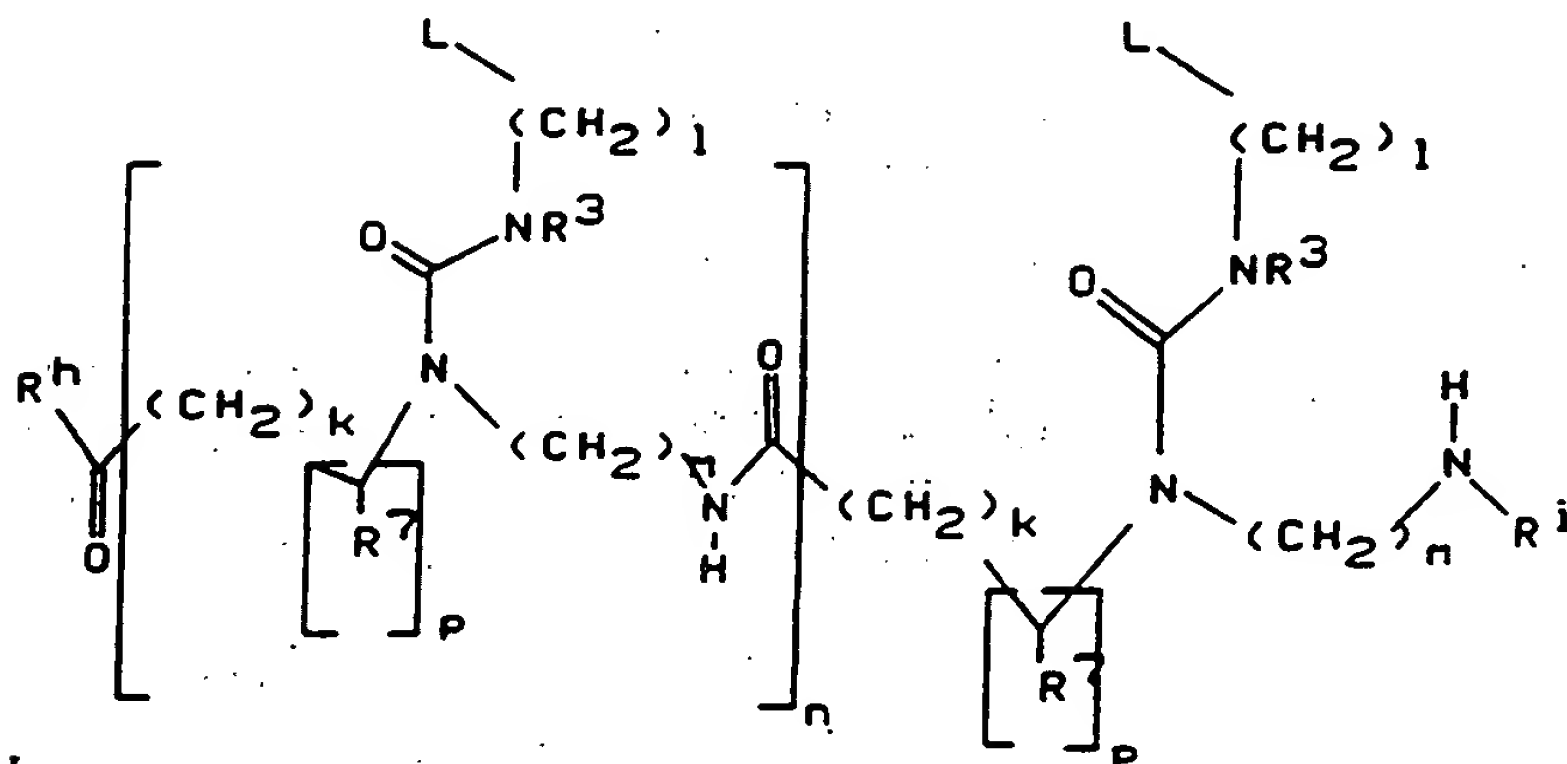
Formula IIc



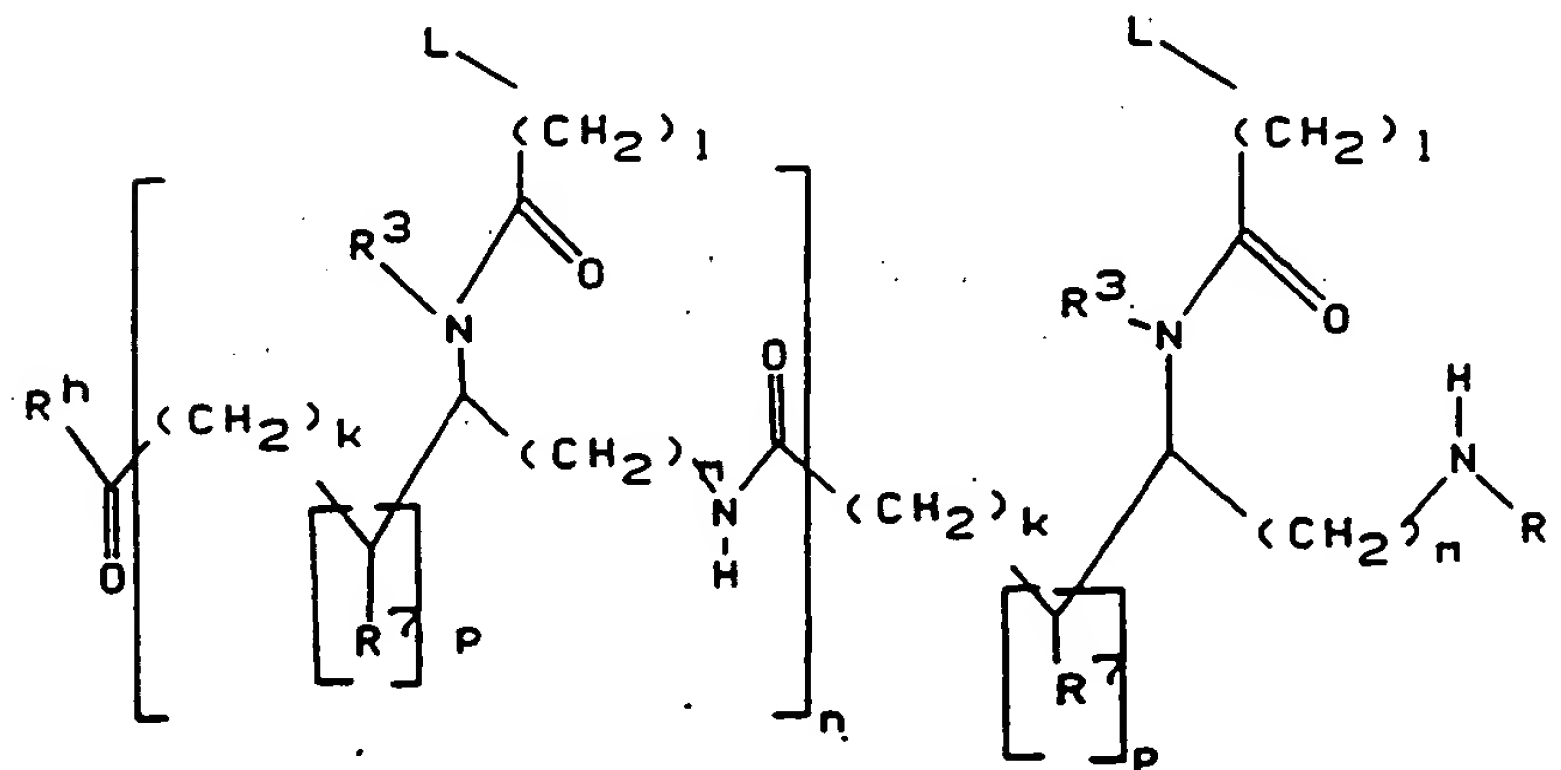
Formula IId



Formula III



Formula IV



Formula V

wherein:

each L is independently selected from the group consisting of hydrogen, phenyl, heterocyclic moieties, naturally occurring nucleobases, and non-naturally occurring nucleobases;

A solid support having a nucleic acid analog bound thereto or capable of capturing such a nucleic acid analog by the techniques described above maybe used to capture from solution a nucleic acid of complementary sequence. A particular virtue of this technique is that one then has the option of removing the captured nucleic acid from the solid support either with or without the nucleic acid analog.

Thus by treating the system with an excess of a chelating agent such as EDTA, the chelated metal can be removed, so freeing the nucleic acid analog, and any hybridized nucleic acid. Alternatively, one may liberate the nucleic acid from the nucleic acid analog on the support by heat or other denaturing methods.

One example of such capture of a nucleic acid would be to hybridize a nucleic acid to the nucleic acid analog capture probe bearing a chelating moiety, and then to capture the resulting complex to a solid bearing metal ions.

When standard DNA probes are used in hybrid selection procedures one of the serious limitations is target sequence inaccessibility due to competing hybridization events. For instance, when targeting double-stranded PCR products the DNA probe competes with the complementary non-target PCR strand. Target sequence inaccessibility can also be caused by secondary and higher order structures in the target nucleic acid. Such structures are well characterized in the case of many metabolically stable RNAs (RNA, tRNA and snRNAs). We have shown that PNA can hybridize to its complementary nucleic acid over a broad range of salt concentrations without loss of affinity and specificity. In fact the affinity of the PNA increases as the salt concentration in the buffer decreases. In theory, this is a most useful property of PNA as it allows hybridization to its target sequence under conditions of low salt that destabilizes normal nucleic acid structures. We have provided an example that this property of PNA can be used to capture a "difficult" oligonucleotide in which the PNA target sequence is designed to form one side of an intra-molecular, perfectly matched 15 bp stem structure.

Methods that facilitate the rapid purification of nucleic acids from complex biological samples are important tools in both basic research and in DNA diagnostics. Compared to methods that rely on physical properties of the nucleic acids for purification, such as density, binding to surfaces, solubility, the hybrid selection method described here offers two main advantages. Firstly, it utilizes a property that is unique to nucleic acids - namely the ability to hybridize to a probe of complementary sequence. Hence, the chance of copurification of other cellular components that may prove inhibitory to downstream applications are likely to be minimal. Secondly, the method allows specific nucleic acids to be targeted thereby removing bulk DNA and RNA that may add to the generation of non-specific background in subsequent target detection procedures.

The invention includes in a third aspect thereof a labeled nucleic acid analog comprising a nucleic acid analog according to the first aspect of the invention, having chelated thereto via said chelating moiety a metal ion as label or having a labeling moiety linked thereto via a metal ion chelated by said chelating moiety.

In the following examples, the PNA used has an amino-ethylglycine backbone and is prepared by the methods specifically described in WO 92/20703. The nomenclature used there in respect of PNAs is used here also.

Example 1

Selective purification of DNA by immobilized histidine tagged PNA

The PNA:

Boc-NH-TG(Z)T.A(Z)C(Z)G(Z).TC(Z)A(Z).C(Z)A(Z)A(Z).C(Z)TA(Z)-CONH-Resin

was constructed. This was extended to the tagged PNA:

H-His₅-NH(CH₂)₅CONH-TGTACGTCACAACTA-NH₂

as follows:

The protected PNA on MBHA resin was coupled with the 6-amino-hexanoic acid linker by boc type solid phase synthesis.

After boc deprotection of the amino terminus, the His₅ motif was built up using an Fmoc strategy. Fmoc-His(Trt)-OH was coupled 2 x 1 h with diisopropylcarbodiimide in DCM/DMF. The Fmoc group was cleaved by treatment with 20 % piperidine in DMF (1 x 5 min and 1 x 10 min). Coupling and Fmoc deprotection were repeated another four times. The trityl protection groups were removed by 50 % TFA in DCM (2 x 30 min). Finally the Z groups were removed and the product cleaved from resin by standard HP procedure. The raw product was purified by preparative HPLC.

The tagged PNA was incubated with either complementary or non-complementary, ³²P labeled oligonucleotides in a 20 µl reaction volume containing 20 mM NaH₂PO₄ (pH 8.0) 0.5M NaCl. Incubation was carried out at room temperature for 15 min. At the end of the incubation period 180 µl of buffer 1 (20 mM NaH₂PO₄ (pH 8.0) 0.5M NaCl) was added and the reaction mixture was loaded onto a Duraphore 0.22 µM spin column (Millipore) packed with 200 µl Ni-NTA-agarose (Pharmacia). The column was centrifuged for 30 seconds at 1000 rpm and the radioactivity in the flow through (named Sup) was counted using a Geiger Muller tube.

The column was washed three times with 200 µl of buffer 1, and the radioactivity in the flow through (named: Wash I-IV) was counted. The column was loaded with 200 µl of buffer 1, incubated at 95°C for 5 min, and centrifuged for 30 seconds at 1000 rpm. The radioactivity in the flow through (named Elu I-II) was counted.

The results are shown in Figure 1. As shown, the non-complementary oligonucleotides (black bars) are all lost from the column during the initial washing steps whereas the complementary oligonucleotide (white bars) remains on the

N,N,N',N'-tetramethyluronium hexafluorophosphate. Ado: 8-amino-3,6-dioxaoctanoic acid. Gly: glycine residue. PNA monomers. PNAD 153 ((His)₆-(ado)₃-TCTCAACAGCGGTAA-NH₂) and PNAD 154 ((His)₆-(ado)₃-GAAGGTAAGTGGCTT-NH₂) were purchased from Biosearch (MA).

The following PNAs were synthesized: PNAD103 (H-TGTACGTCACAACATA-NH₂), PNAD106 (H-(ado)₃-TGTACGTCACAACATA-NH₂), PNAD 111A ((His)₆-(ado)₃-TGTACGTCACAACATA-Gly-NH₂), PNAD113 ((His)₆-(ado)₃-GATCCTGTACGTCACAACATA-Gly-NH₂), PNAD133 ((His)₆-(ado)₃-GGCTGCAGGAATTCGA-Gly-NH₂), and derivatives of PNAD111 containing 3 from 3 to 8 histidine residues. The PNA segments including the ado linkers were synthesized manually by the improved solid phase PNA synthesis method which follows the Boc-strategy. Couplings were performed by adding HBTU to the PNA monomers, and acetic anhydride was used as the capping agent. The Boc protecting groups were removed by adding TFA/m-Cresol (95/5). The histidine segments were synthesized using the Fmoc strategy and the molar ratio of Fmoc-His(Trt)-OH/diisopropylcarbodiimide was the same as the ratio of PNA monomers/HBTU during the PNA segment syntheses. DMF/DCM was used during couplings and deprotection was performed by adding 20 % piperidine in DMF (2 x 10 min). During the polyhistidine synthesis couplings were not followed by capping. Finally, the trityl protection groups were removed by TFA treatment (3 x 30 min) and the PNAs were deprotected and cleaved from the resin by the standard low-high TFMSA procedure. The crude PNAs were purified by reversed phase HPLC.

Selection of target DNA

5 µl of the His-PNA probes (5 OD₂₆₀/ml) were mixed with 10 µl of ³²P labeled DNA oligonucleotide (0.2 µM) or 10 µl of ³²P labeled, in vitro transcribed RNA, 50 µl of 8M urea, 100 µl of selection buffer (20 mM, Na₂HPO₄) (pH 8.0), 500 mM NaCl and 0.1 % Triton X-100) and 35 µl of water in an Eppendorf tube. The solution was heated to 95°C for 5 min and incubated for 10 min in a heating block at the indicated temperature. Meanwhile, a 400 µl sample of Ni-NTA resin (Quiagen) was loaded onto an Eppendorf spin column (Dura-pore 0.45 µm; Millipore) and centrifuged at 200 rpm for 30 sec to remove the Ni-NTA storage buffer. The column was washed three times in 200 µl water and equilibrated in 200 µl selection buffer. At the end of the hybridization period the mixture was loaded onto the column and centrifuged at 200 rpm for 30 sec. The column was washed several times in 200 µl of selection buffer to remove non-specifically bound nucleic acids. Finally, the purified target nucleic acids were eluted from the column by 1) adding 200 µl of selection buffer to the column, 2) incubating the column in a heating block at 95°C for 5 min and 3) centrifugation at 200 rpm for 30 sec.

Radioactivity in the column fractions was counted using either a Geiger-Muller counter or a scintillation counter. Where analysis was conducted by gel electrophoresis, the nucleic acid in the column fractions was precipitated by adding 5 µg of carrier tRNA, 1 volume of 4M ammonium acetate and 2 volumes of 96 % ethanol. The precipitated nucleic acid was recovered by centrifugation at 20000 rpm

the PNAs were hybridized separately to oligonucleotides containing either a complementary or a single base mismatched PNA target site and the melting temperature of each duplex (T_m) was determined spectrophotometrically. To mimic the situation normally encountered in hybridization experiments (i.e. probe target site is part of a large nucleic acid) the oligonucleotides were synthesized as 40mers comprising the 15nt PNA target site in the middle. As shown in Figure 2 neither the addition of the ado linkers nor the His₆ tag have any significant effect on the affinity or the specificity of the PNA. Thus, the duplexes formed between the fully complementary oligonucleotide and either of the three PNAs have essentially similar T_m values. Likewise the T_m values for the various single se mismatched complexes are quite similar.

Capture efficiency is affected by the number of histidine residues carried out by the PNA

For optimal capture efficiency the PNA must carry sufficient histidine residues to provide tight binding to the Ni-NTA resin. To determine the number of histidine residues that would provide optimal capture, derivatives of PNAD111 carrying from 3 to 8 histidines were synthesized and analyzed as above using the complementary, labeled 40mer oligonucleotide. As shown in Figure 2, capture efficiencies improve up to 5 to 6 histidine residues. Increasing the number of histidine residues further to 7 or 8 does not increase the capture efficiency. Thus, the His₆ tag chosen originally was used in all further experiments.

His₆ PNA allows the selection of oligonucleotides in which the target sequence is part of an intra-molecular stem structure

It has been shown previously (Nature 365, 566-568 (1993)) that the thermostability of a PNA/DNA duplex increases slightly as the ionic strength of the buffer was decreased from 100 mM to 10 mM Na⁺. When used as a hybrid selection probe, this property of PNA is most useful as it allows hybridization under conditions of very low salt that selectively destabilize nucleic acid structures that could interfere with probe binding. The published analysis was limited to a fully complementary PA/DNA duplex and therefore did not address the question of whether the specificity of PNA/DNA duplex formation was maintained under low salt conditions. To assess this, we measure the T_m values of fully matched and single base mismatched His₆-PNA/DNA duplexes at various salt concentrations. As shown in Figure 3 we confirmed the previously reported increase in affinity of the fully complementary PNA/DNA duplex as the salt concentration was lowered. Thus, within the Na⁺ concentration range employed (1 to 540 mM) we observed an increase in affinity of approx. 12°C. Similar behavior was observed with the duplexes containing single base mismatches showing that the specificity of the PNAs was retained over the entire range of ionic strength tested. To illustrate the destabilization of nucleic acid structures at similar salt conditions, Figure 3 includes the T_m values for the corresponding PNA/DNA duplexes. At a concentration of 2 mM Na⁺ the complementary DNAs did not produce melting curves within a 10 - 90°C temperature range, indicating that no hybridization takes place.

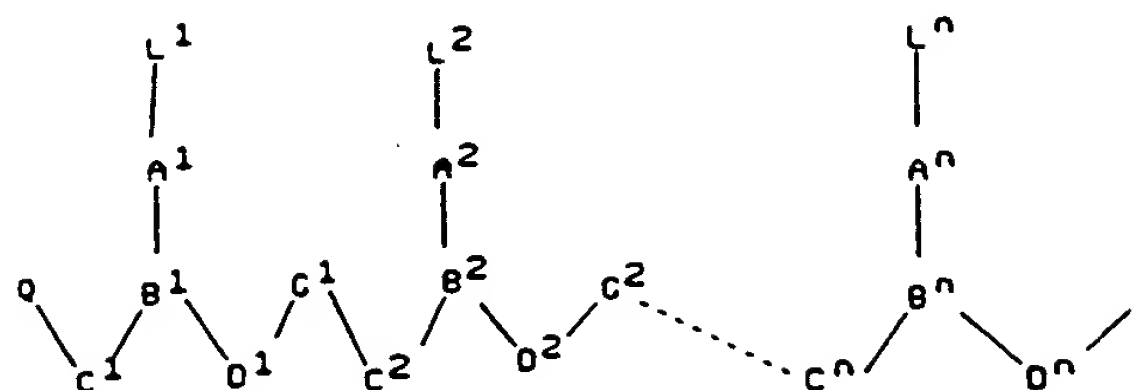
shown in Figure 6 essentially no RNA is captured on the column in the reactions where either the 290nt. control RNA is used in combination with non-complementary (His)₆-PNAs (row 1 and 2) or where the (His)₆-PNAs are excluded (row 3 and 7). in contrast, specific capture is observed when the 257nt. and 2224nt. RNAs are incubated with the complementary (His)₆-PNAs. In the case of the 257nt. RNA the observed capture efficiency is approx. 45 % when using single (His)₆-PNAs (row 4 and 5).

The capture efficiency decreases as the size of the target RNA increases. Thus, PNAD111 which is complementary to both RNAs is about twice as effective in selecting the 257nt RNA (44.0 %, row 4) as the 2224nt RNA (25 %, row 8). Similar differences in capture efficiencies of the 257 and 2224nt RNA transcripts is obtained with the majority of other PNAs tested (PNAD133: row 5; PNAD113, row 9; and PNAD154, row 11). The PNAD 153 (row 10), however, is about 50 % more effective in capturing the 2224 RNA transcript as compared to the other PNAs. The reason for this increased capture efficiency is unclear.

The location of the PNA target site in the RNA does not appear to affect capture efficiencies significantly. Thus, PNAD111 (row 8) and PNAD154 (row 11) whose target sequences are located at the end and in the middle of the 2224 RNA transcript, respectively, are equally efficient in capture and this also applies to the two PNAs (PNAD111, row 4 and PNAD133, row 5) directed against the 257nt. RNA transcript.

The size of the PNA domain in the (His)₆-PNA chimera does not appear to affect the capture efficiency. Thus, PNAD111 (a 15mer PNA) is as good in selecting the 2224nt RNA as its 5 base extended 20mer derivative, PNAD113 (compare row 8 and 9). This suggests that the weak link in the selection procedure is either the link between the (His)₆ segment and the chelated Ni²⁺ ion or the link between the Ni²⁺ ion and the NTA molecule on the resin. This contention is supported by the finding that capture efficiencies improve when two or three (His)₆-PNAs are used in conjunction, thereby providing more attachment points for the PNA/RNA complex to the Ni-NTA resin. Using for instance 3 different (His)₆-PNAs the capture efficiency of the 2224nt RNA increases to 64.9 % (row 15) as compared to approx. 25 % when using each (His)₆-PNA separately.

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Formula 1

wherein:

n is at least 2,

each of L^1-L^n is independently selected from the group consisting of hydrogen, hydroxy, (C_1-C_4) alkanoyl, naturally occurring nucleobases, non-naturally occurring nucleobases, aromatic moieties, DNA intercalators, nucleobase-binding groups, heterocyclic moieties, reporter ligands and chelating moieties;

each of C^1-C^n is $(CR^6R^7)_y$ where R^6 is hydrogen and R^7 is selected from the group consisting of the side chains of naturally occurring α amino acids, or R^6 and R^7 are independently selected from the group consisting of hydrogen, (C_2-C_6) alkyl, aryl, aralkyl, heteroaryl, hydroxy, (C_1-C_6) alkoxy, (C_1-C_6) alkylthio, NR^3R^4 and SR^5 , where R^3 and R^4 are as defined below, and R^5 is hydrogen, (C_1-C_6) alkyl, hydroxy, alkoxy, or alkylthio-substituted $(C_1$ to $C_6)$ alkyl or R^6 and R^7 taken together complete an alicyclic or heterocyclic system;

each of D^1-D^n is $(CR^6R^7)_z$ where R^6 and R^7 are as defined above;

each of y and z is zero or an integer from 1 to 10, the sum $y + z$ being from 2 to 10;

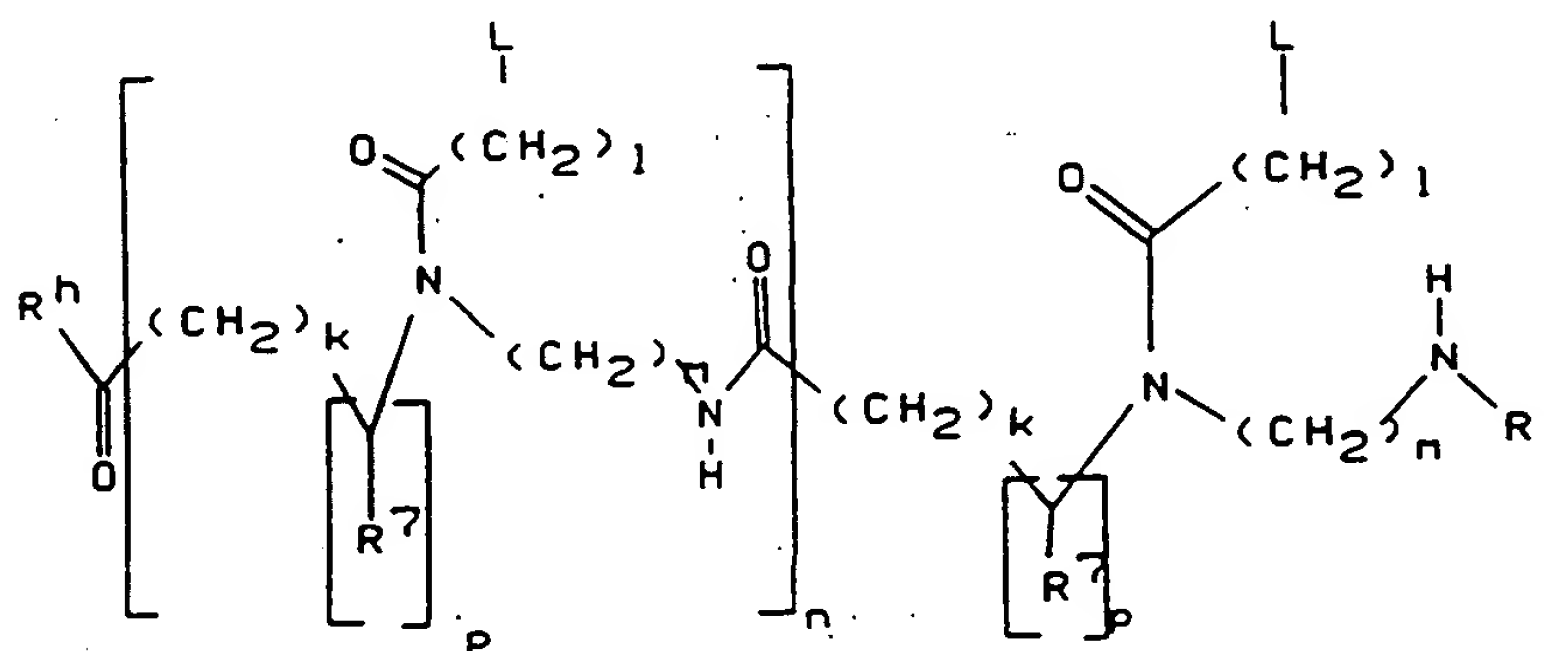
each of G^1-G^{n-1} is $-NR^3CO-$, $-NR^3C^5-$, $-NR^3SO-$ or $-NR^3SO_2-$, in either orientation, where R^3 is as defined below;

each of A^1-A^n and B^1-B^n are selected such that:

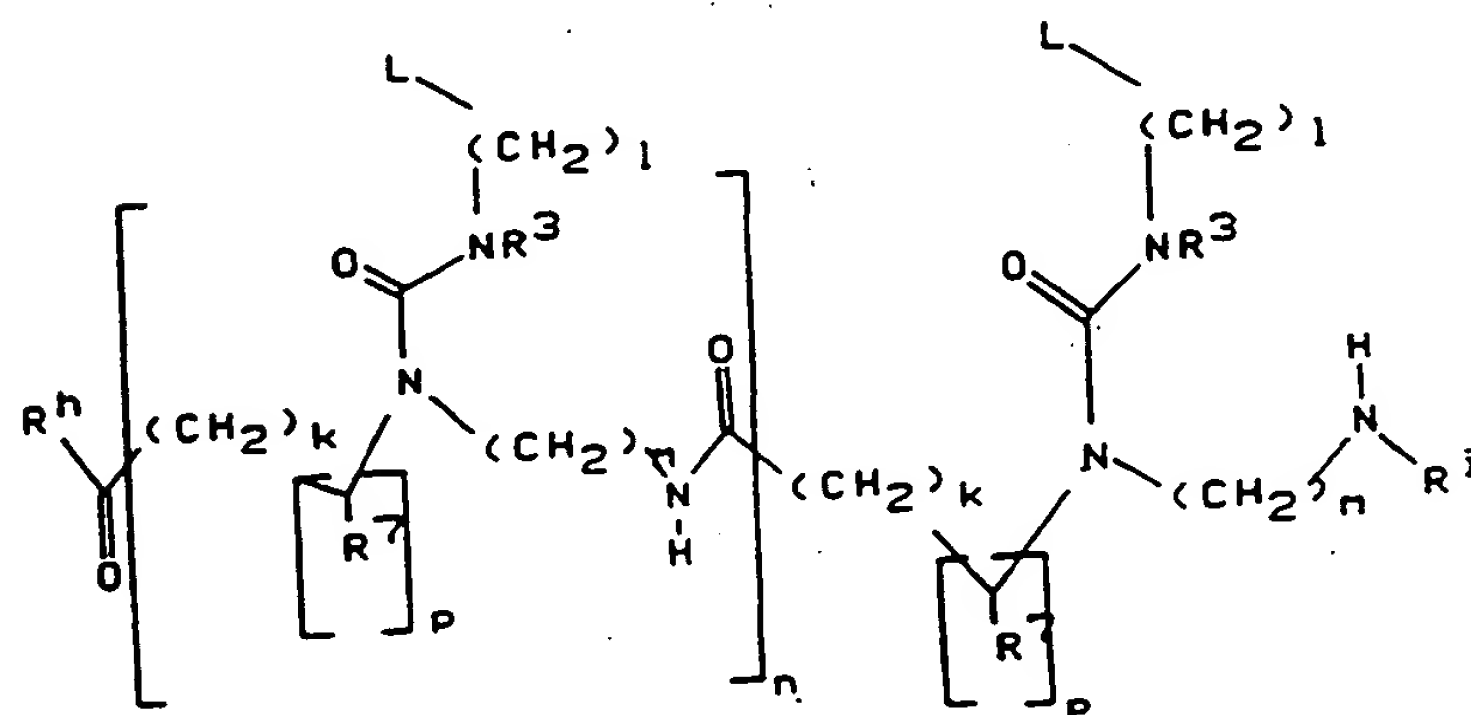
- (a) A is a group of formula (IIa), (IIb), (IIc) or (IId), and B is N or R^3N^+ ; or
- (b) A is a group of formula (IId) and B is CH ;

oligodeoxyribonucleotides, oligonucleosides and soluble and non-soluble polymers, and -R''' is a chelating moiety.

12. A nucleic acid analog as claimed in Claim 11, wherein said nucleic acid analog comprises a compound of the general formula III, IV or V:



Formula III



Formula IV

16. A method of concentrating a solution of a nucleic acid analog according to any one of claims 1 to 13, comprising capturing said nucleic acid analog from a first volume of solution by a method as claimed in claim 14 or claim 15, removing the solid support and captured nucleic acid from said solution and eluting the nucleic acid analog from the solid support in a quantity of liquid such as to produce a second volume of a solution of said nucleic-acid analog which is less than said first volume of solution.
17. A labeled nucleic acid analog comprising a nucleic acid analog as claimed in any one of claims 1 to 13, having chelated thereto via said chelating moiety a metal ion as label or having a labeling moiety linked thereto via a metal ion chelated by said chelating moiety.
18. A labeled nucleic acid analog as claimed in claim 17, wherein said metal ion is a radio label or a fluorescent label.
19. A labeled nucleic acid substantially as herein before described in Example 2 or Example 3.
20. A method of capturing a nucleic acid substantially as herein before described in Example 1.

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Fig. 1

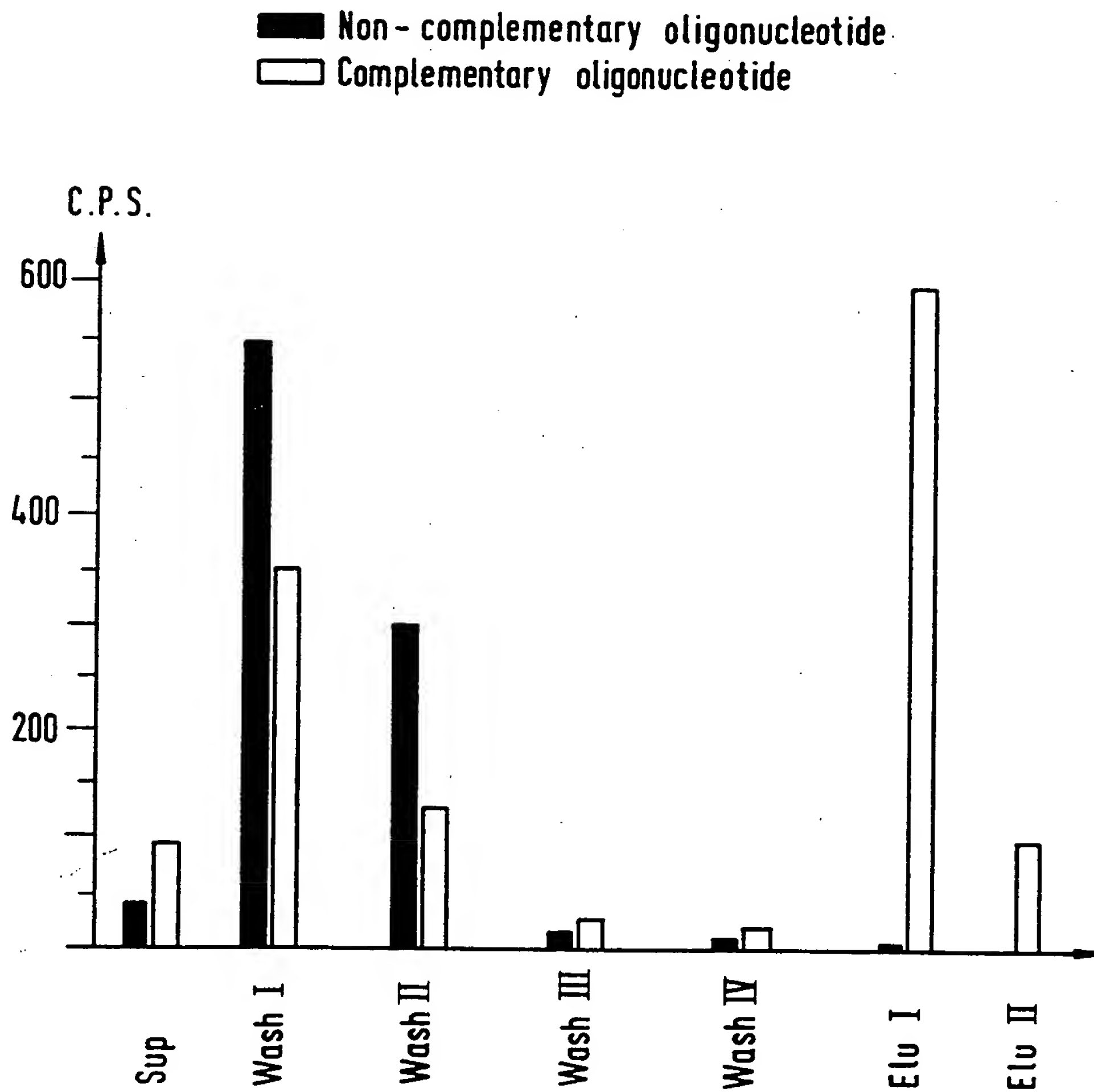


Fig. 2

PNA / DNA COMPLEX	Mismatch	X = none	X = ado ₃	X=ado ₃ -His ₆
5'-CTAGAGGATCTAGTTGTGACGTACAGGATCTTTTTCATAG-3' PNA: H ₂ N-ATCAACACTGCCATGT-X	None	64.2 °C	65.2 °C	63.6 °C
5'-CTAGAGGATCTAGTTGTGAACTACAGGATCTTTTTCATAG-3' PNA: H ₂ N-ATCAACACTGCCACGT-X	G _{PNA} /A _{DNA}	49.0 °C	49.6 °C	47.4 °C
5'-CTAGAGGATCTAGTTGTGATGTACAGGATCTTTTTCATAG-3' PNA: H ₂ N-ATCAACACTGCCATGT-X	G _{PNA} /T _{DNA}	56.4 °C	57.8 °C	56.2 °C
5'-CTAGAGGATCTAGTTGTGAGGTACAGGATCTTTTTCATAG-3' PNA: H ₂ N-ATCAACACTGCCATGT-X	G _{PNA} /G _{DNA}	50.0 °C	51.6 °C	50.6 °C

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Fig. 3

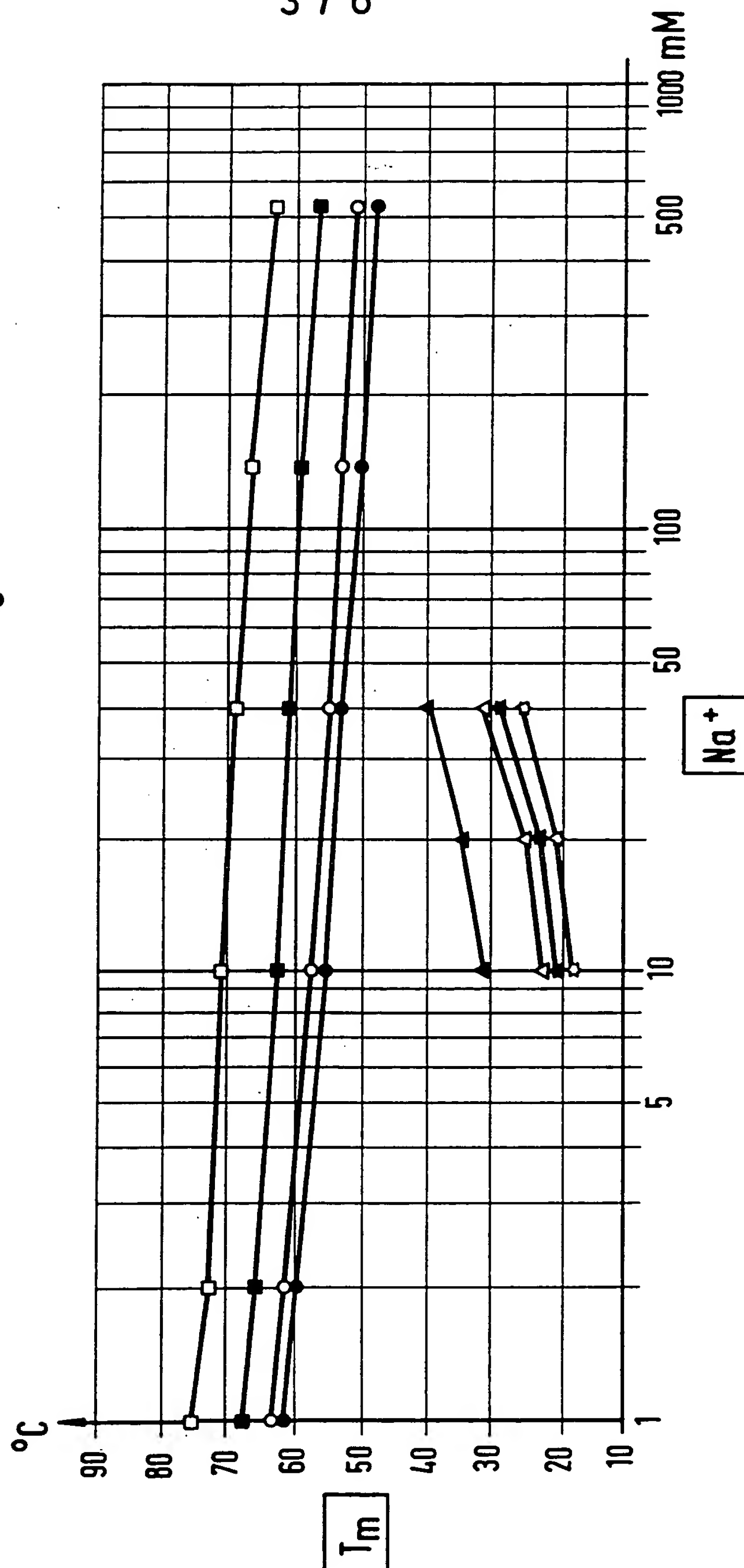


Fig. 4B

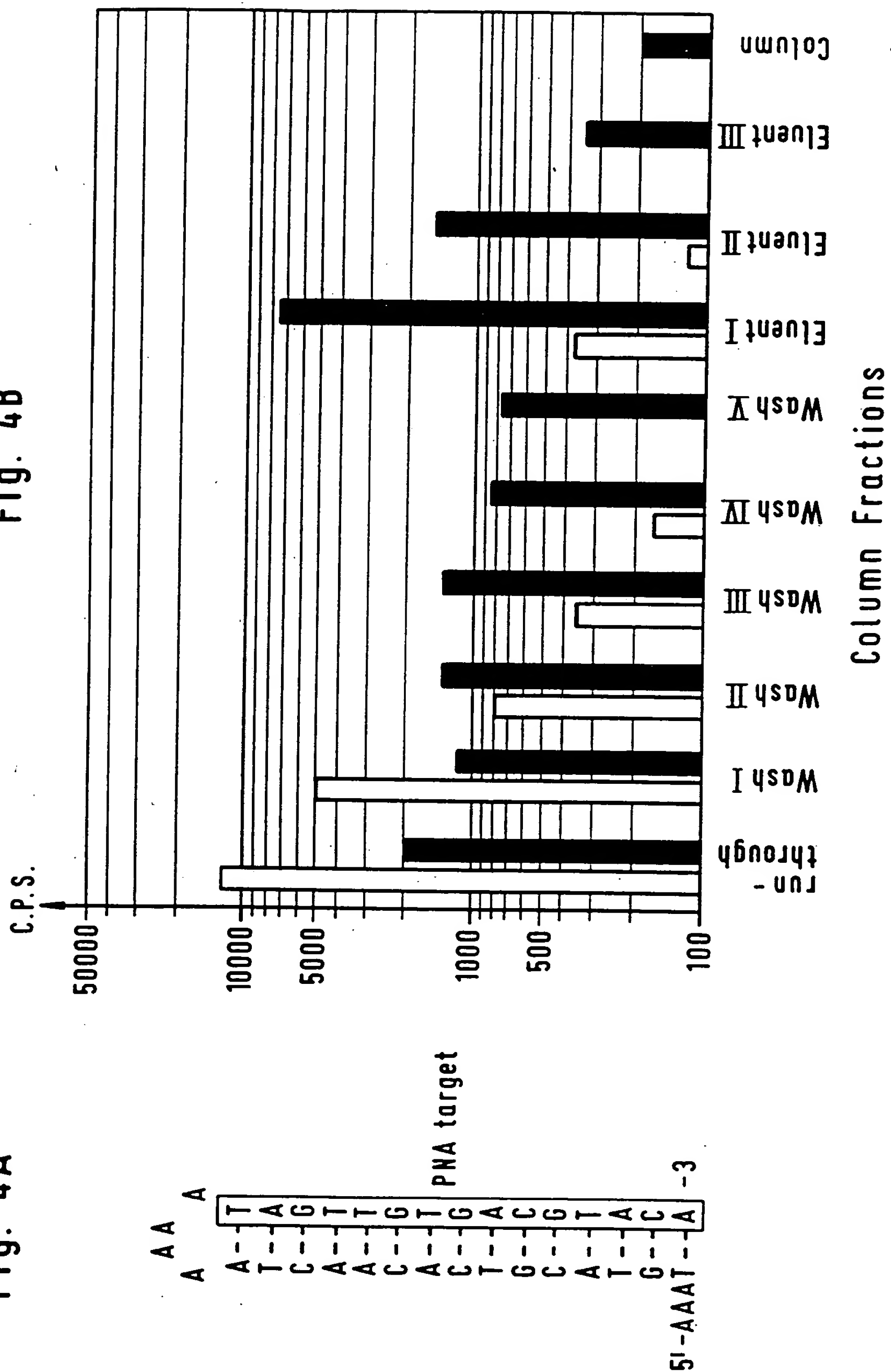
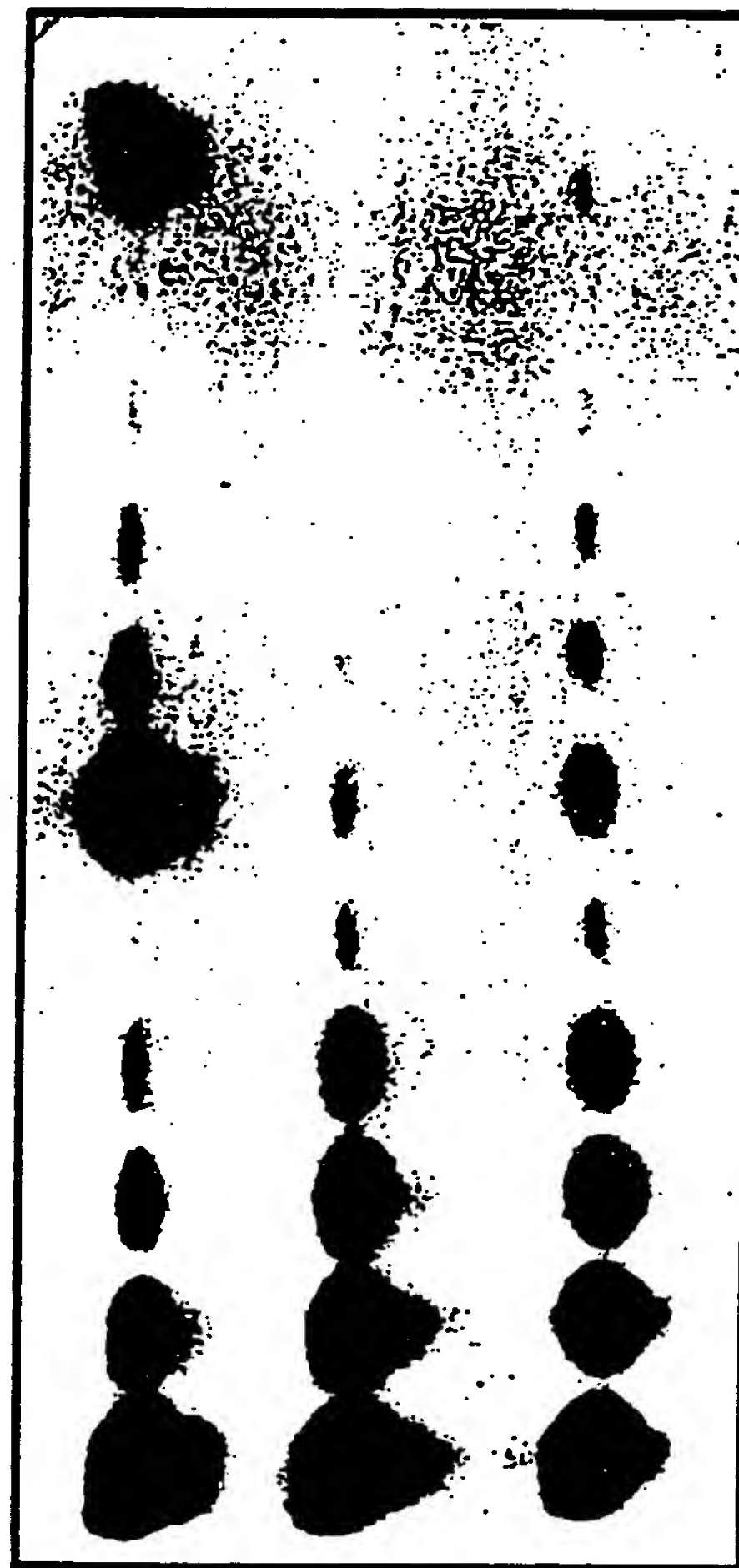


Fig. 5

Selection round I Selection round II

Starting material Run - through Wash I Wash II Wash IV Eluent I Run - through Wash I Wash II Wash IV Eluent II



Complementary
oligonucleotide

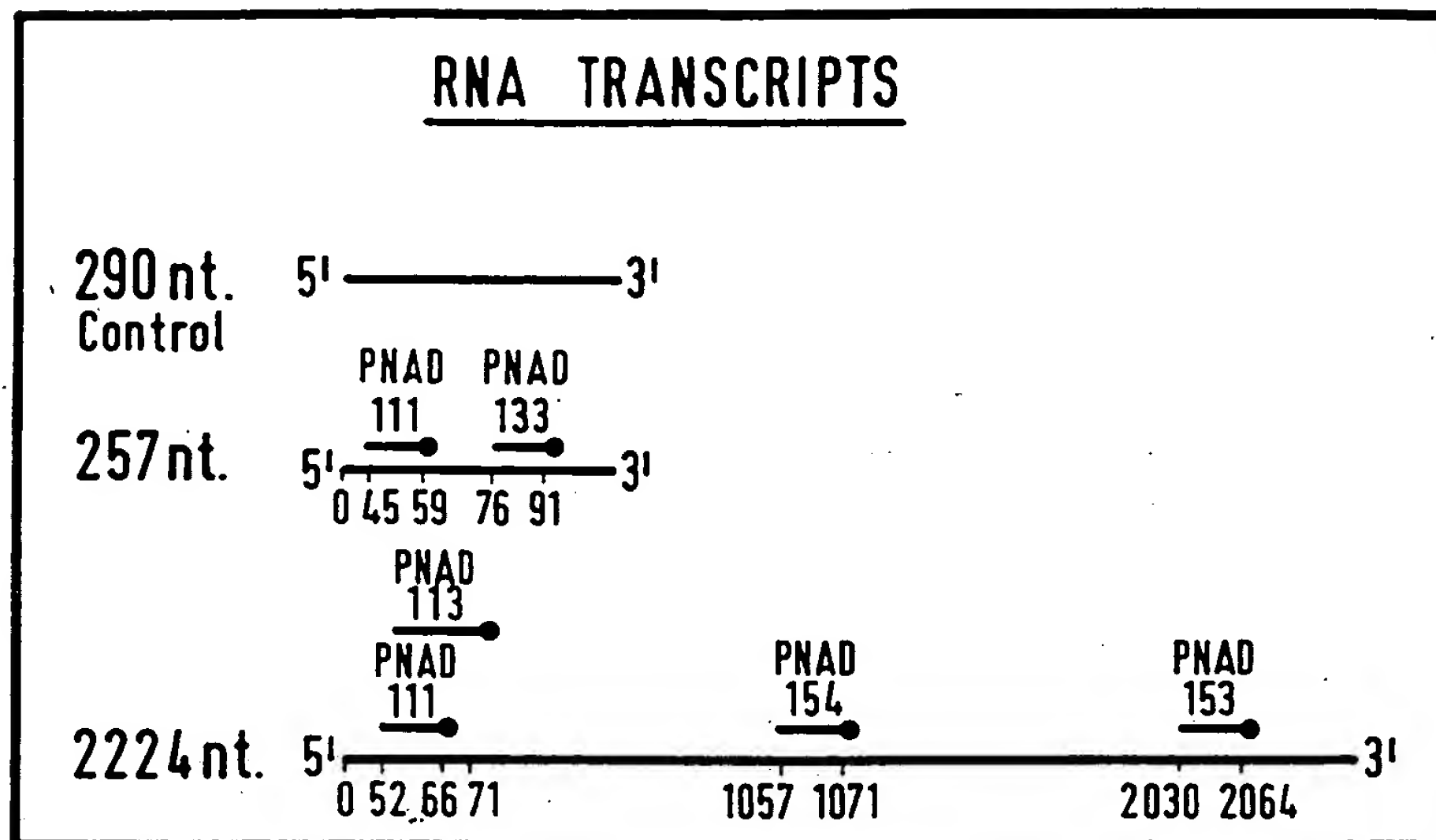
Non-complementary
oligonucleotide

Mismatched
oligonucleotide

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Fig. 6

A



B

Row	Transcript size	PNA	Size of PNA segment	% in eluent	% Remaining on column
1	290 nt.	111	15 mer	2.0	1.0
2	290 nt.	113	20 mer	1.7	0.6
3	257 nt.	none	-	1.4	0.1
4	257 nt.	111	15 mer	44.0	0.7
5	257 nt.	133	16 mer	45.2	0.8
6	257 nt.	111, 133	-	57.4	0.7
7	2224 nt.	none	-	3.7	1.7
8	2224 nt.	111	15 mer	25.0	2.6
9	2224 nt.	113	20 mer	26.7	3.3
10	2224 nt.	153	15 mer	38.3	1.9
11	2224 nt.	154	15 mer	22.1	1.4
12	2224 nt.	111, 153	-	53.1	3.9
13	2224 nt.	111, 154	-	47.7	3.5
14	2224 nt.	153, 154	-	50.9	4.1
15	2224 nt.	111, 153, 154	-	64.9	3.8